Transcription factors Mash-1 and Prox-1 delineate early steps in differentiation of neural stem cells in the developing central nervous system

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SUMMARY

Like other tissues and organs in vertebrates, multipotential stem cells serve as the origin of diverse cell types during genesis of the mammalian central nervous system (CNS). During early development, stem cells self-renew and increase their total cell numbers without overt differentiation. At later stages, the cells withdraw from this self-renewal mode, and are fated to differentiate into neurons and glia in a spatially and temporally regulated manner. However, the molecular mechanisms underlying this important step in cell differentiation remain poorly understood. In this study, we present evidence that the expression and function of the neural-specific transcription factors Mash-1 and Prox-1 are involved in this process. In vivo, Mash-1- and Prox-1-expressing cells were defined as a transient proliferating population that was molecularly distinct from self-renewing stem cells. By taking advantage of in vitro culture systems, we showed that induction of Mash-1 and Prox-1 coincided with an initial step of differentiation of stem cells. Furthermore, forced expression of Mash-1 led to the down-regulation of nestin, a marker for undifferentiated neuroepithelial cells, and up-regulation of Prox-1, suggesting that Mash-1 positively regulates cell differentiation. In support of these observations in vitro, we found specific defects in cellular differentiation and loss of expression of Prox-1 in the developing brain of Mash-1 mutant mice in vivo. Thus, we propose that induction of Mash-1 and Prox-1 is one of the critical molecular events that control early development of the CNS.

Key words: Neural development, Stem cell, Neuron, Glia, Mash-1, Prospero

INTRODUCTION

During development in vertebrates, undifferentiated cells, termed progenitors or precursors, undergo successive commitment and differentiation, thereby generating more restricted cell types. Among them, stem cells are a specialized group of cells that serve as the ultimate origin of multiple cell types constituting a particular tissue or organ (Hall and Watt, 1989). They are capable of continuing self-renewal without manifesting features of terminally differentiated cells, thus often maintain multipotentiality. At particular stages or timings of development, stem cells commit to differentiation pathways leading to the generation of multiple cell types. Thus, one of the central issues in developmental cell biology is to understand the molecular and cellular mechanisms underlying the cell-fate transition of stem cells between self-renewal and differentiation.

The developing neuroepithelium is the primordia of the mammalian central nervous system (CNS) that gives rise to all three major cell types present in the adult brain, including neurons, astrocytes and oligodendrocytes (McConnell, 1995; McKay, 1997). Recent lineage-tracing studies in vivo (Cepko et al., 1995) and clonal culture experiments in vitro (Reynolds et al., 1992; Kilpatrick and Bartlett, 1993; Davis and Temple, 1994; Williams and Price, 1995; Qian et al., 1998) have demonstrated that a population of these cells have stem cell properties. In the early forming neuroepithelium, stem cells continue proliferation and increase their total cell numbers without overt differentiation. At subsequent stages, however, the cells withdraw from this self-renewal mode and begin to commit to generating both neurons and glia. This lineage commitment and differentiation of stem cells proceeds with distinct kinetics and manner among different areas and/or domains, finally contributing to the highly organized and
complex morphogenesis of the brain. Thus, cell-fate determination of neural stem cells and subsequent successive steps of differentiation should be under strict control at the molecular and cellular levels; details of this, however, remain poorly understood.

An apparent complexity of mammalian neural development is that the processes of neurogenesis and gliogenesis involve multiple types of intermediate precursors, suggested by recent studies of other experimental organisms. In Drosophila, for example, neuroectodermal stem cells, termed neuroblasts, give rise to secondary precursor cells called ganglion mother cells (GMCs), which further divide into postmitotic neurons and/or glia (Jan and Jan, 1993). The presence of transient precursor populations has also been demonstrated during development of the peripheral nervous system (PNS) (Lo and Anderson, 1995; Gordon et al., 1995). Consistently, recent studies have revealed that the developing neuroepithelium is also composed of heterogeneous cell types (Kilpatrick et al., 1995; Temple and Qian, 1996; Lillien, 1998; Qian et al., 1998). Furthermore, many types of epigenetic signals have been shown to influence lineage commitment and differentiation of various CNS cell types (see McKay, 1997 for a detailed review). However, it is not yet precisely determined what cell types are primary targets for these signaling factors, mainly because of the complexity of lineage relationships among heterogeneous precursors. In order to solve this cellular complexity and to clarify the underlying molecular mechanisms, it is particularly important to study intrinsic regulatory molecules that function in distinct precursor cells. We have therefore searched for molecular markers that can be used to identify and characterize transient precursor cell types during neurogenesis of the CNS.

In this study, we have focused upon the two genes Mash-1 and Prox-1, expressed in the early developing brain. Mash-1 is a mammalian homolog of the proneural genes of the Drosophila achaete-scute complex (AS-C), which encodes a basic helix-loop-helix (bHLH)-type transcription factor (Johnson et al., 1990). The fly AS-C genes are expressed in neuroblasts as well as in the secondary precursor GMCs, and play key roles in multiple steps of neurogenesis (Jan and Jan, 1993). Likewise, Mash-1 has been found to be expressed in the developing PNS (Lo et al., 1991), and plays an essential role for the development of autonomic neurons (Guillemot et al., 1993; Sommer et al., 1995). Although Mash-1 is also widely expressed in the developing CNS (Lo et al., 1991; Guillemot and Joyner, 1993), no obvious histological anomalies have been detected in the brain of Mash-1 knock-out mice, except for a subset of olfactory neurons and noradrenergic neurons in the brainstem (Guillemot et al., 1993; Cau et al., 1997; Hirsch et al., 1998). Thus, it is not yet understood how Mash-1 is involved in neurogenesis in the CNS. Prox-1, the other marker analyzed in this study, encodes a homeobox protein that is structurally homologous to Drosophila prospero (Oliver et al., 1993). In fly neurogenesis, expression of prospero depends on bHLH genes of the AS-C in neuroblasts and GMCs, and it is involved in subsequent specification of neuronal progeny (Doe and Technau, 1993; Hirata et al., 1995). Although a previous study reported a dynamic expression profile of Prox-1 in the developing brain (Oliver et al., 1993), its regulation and function still remain unknown.

Here we have examined spatial and temporal expression patterns of Mash-1 and Prox-1 proteins in the CNS with particular emphasis upon their correlation with early steps in the differentiation of neural stem cells. In the developing forebrain and spinal cord, the cells expressing these proteins transiently appeared as proliferating precursors that were distinct from self-renewing stem cells. We used in vitro culture systems to demonstrate that induction of Mash-1 and Prox-1 specifically coincided with the time at which stem cells ceased self-renewal and began to differentiate into neurons. Furthermore, by using a stem cell-derived cell line, we showed that forced expression of Mash-1 down-regulated the undifferentiated neuroepithelial cell marker nestin and concomitantly induced expression of Prox-1. Consistent with these studies in vitro, we found specific defects in differentiation of stem cells and loss of Prox-1 expression in the brain of Mash-1 mutant mice in vivo. Taken together, our results demonstrate that Mash-1 and Prox-1 delineate early steps in differentiation of neural stem cells in the developing central nervous system.

MATERIALS AND METHODS

Immunostaining and in situ hybridization studies
The antibodies against Mash-1 and Dlx-1 (kind gifts from Drs D. Anderson and J. Rubenstein, respectively) were described previously (Lo et al., 1991; Porteus et al., 1994). The affinity-purified anti-Prox-1 rabbit antibody was prepared by immunization with a synthetic oligopeptide NH2-Lys-Ser-Pro-Asp-Cys-Leu-Gln-Leu-Leu-His-Glu-COOH, corresponding to the predicted C-terminal amino acid sequence of murine Prox-1 (Olive et al., 1993). Our preliminary study confirmed that the cDNA for rat Prox-1 encodes exactly the same sequence at its C terminus (T. M. and M. N., unpublished). The expression of Mash-1, Prox-1 and other markers in the developing CNS was examined by immunocytochemistry. Sprague-Dawley rat embryos were fixed by immersion in freshly prepared 4% paraformaldehyde in phosphate-buffered saline (PBS) for 2-4 hours at 4°C, depending on their ages. To label proliferating cells, 50 mg of 5-bromo-2′deoxyuridine (BrdU) dissolved in sterile PBS was injected intraperitoneally into pregnant rats 3 hours before they were killed. Immunoperoxidase staining was performed with a combination of biotinylated anti-mouse IgG antibody or anti-rabbit IgG antibody (Chemicon) and avidin-biotinylated peroxidase complex (Vector). Staining was visualized with an ImmunoPure cobalt/nickel-enhanced dianaminobenzidine Substrate Kit (Pierce). For double-staining of BrdU with other antigens, sections were first reacted with primary antibodies and alkaline phosphate (AP)-conjugated antibodies (Vector), and visualized with an AP Substrate Kit IV (Vector). Subsequently, the slides were treated with a 2 M solution of hydrochloric acid for 20 minutes at room temperature, and subjected to staining with peroxidase-conjugated anti-BrdU monoclonal antibody (Boehringer). To count cell numbers, cell nuclei were stained with 2 µg/ml bis-benzimide (Molecular Probe) for 5 minutes just before mounting. Identification of different histological zones within the developing neuroepithelium followed the definitions by the Boulder Committee (1970) and Bhidie (1996). The ventricular zone was identified as the region containing bipolar cell bodies that were radially aligned relative to the ventricular surface of the neural tube, which was revealed by nestin staining. On the other hand, the subventricular zone cells were more densely packed and randomly oriented than those in the VZ, many of which were heavily labeled with BrdU. The mantle zone was defined as the region where MAP2-positive neurons resided.

Nonradioactive whole-mount in situ hybridization to dissected brain samples was performed according to Osumi et al. (1997).
Digoxigenin-labeled antisense probes for rat Mash-1, Pax-6 and Dlx-1 were described in Nakagawa et al. (1996). The staining was visualized with alkaline phosphatase-conjugated anti-digoxigenin Fab fragment (Boehringer) with its color-indicating substrate.

Wild-type and Mash-1 mutant mouse embryos were obtained from intercrosses of Mash-1+/− mice (Guillenot et al., 1993), and subjected to immunohistochemical studies as described above.

Indirect immunocytochemical analyses of cultured cells were performed as described previously (Nakafuku and Nakamura, 1995). Fluorescein isothiocyanate (FITC)- and Texas Red (TR)-conjugated secondary antibodies (Amersham) were used for double staining. In previous studies, we confirmed that the R24 anti-GD3 ganglioside antibody specifically stained oligodendrocyte precursors, but not nestin+ or GFAP+ cells under our culture conditions.

**Primary culture of neuroepithelial cells**

Neuroepithelia of the forebrain of E11.5 and E14.5 rat embryos were dissected out (E9.5 was defined as the day on which the corporative plug was found), and the cells were subsequently cultured either of the following two conditions as described (Nakagawa et al., 1996). In monolayer culture, the cells were seeded onto poly-D-lysine (100 μg/ml-coated chambers (Nunc) at a density of 2.5×10⁶ cells/cm² in a standard medium [1:1 mixture of a Dulbecco’s modified Eagle’s medium (DMEM) and F-12 medium (Sanko Junyaku)] with 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma) containing the B-27 culture supplement (Gibco). The second culture condition is termed aggregation culture, in which the cells were maintained in suspension at a density of 1×10⁵ cells/ml in the above standard medium supplemented with 10% fetal bovine serum (FBS; Sanko Junyaku), 5% horse serum (HS; Gibco), 20 ng/ml basic fibroblast growth factor (bFGF; Boehringer) and 5 ng/ml epithelial growth factor (EGF; Sigma). Culture dishes were coated with poly(2-hydroxy-ethyl methacrylate) (polyHEMA; Sigma, 1.6 mg/cm²) to prevent cell attachment. Under these conditions, the cells were allowed to form aggregates, herein termed neurospheres. To continue culture for more than 4 days, a half volume of the medium was refreshed every 3 days.

At subsequent steps, the aggregates were either re-seeded onto poly-D-lysine-coated dishes and maintained in monolayer described above, or were dissociated into single cells by trypsinization and subjected to additional cycles of aggregation cultures. Under the above conditions, numbers of viable cells were determined by staining with Trypan Blue (Sigma).

**Culture and manipulation of MNS-70 cells**

The neural stem cell line MNS-70 was established and maintained as described previously (Nakagawa et al., 1996). The cell line was immortalized by the c-mycer gene, which encodes a fusion product of c-Myc and the ligand-binding domain of the estrogen receptor. With this system, it is possible to conditionally activate the c-Myc protein by adding estrogen (e.g., β-estradiol) to the culture medium (Nakafuku and Nakamura, 1995). To induce differentiation, MNS-70 cells were subjected to aggregation culture for 3 days in the standard medium containing 20 ng/ml bFGF and 1 μM β-estradiol. Cell aggregates were then re-seeded onto poly-D-lysine-coated dishes, and cultured in differentiation medium (standard medium supplemented with 10% FBS) for 3 days (differentiation culture). Sublines of MNS-70 cells that constitutively expressed Mash-1 were established by transfection with a Mash-1-expression plasmid by the standard calcium precipitation method. The plasmid harbored the 2.3 kb rat Mash-1 cDNA (a kind gift from Dr. Kagayama, Kyoto University) under the control of the Molony sarcoma virus long-terminal repeat and the hygromycin-resistant gene. Transfected cells were cultured in a medium containing 0.3 mg/ml of hygromycin B (Sigma), and were cloned during the following 4-6 weeks. Each single clone was independently expanded and subjected to further analyses. Hygromycin B was included in the medium to maintain the clones in monolayer, but was omitted when the cells were induced to differentiate. We observed essentially identical expression patterns of Mash-1, Prox-1 and other protein markers in those clones irrespective of the presence or absence of Hygromycin B.

**Western blot analysis**

Expression patterns of Mash-1, Prox-1 and nestin were analyzed by western blotting as described (Nakagawa et al., 1996). The cells cultured under different conditions were harvested, washed twice with PBS, and lysed directly in Laemmli’s sample buffer. Equal amounts of total proteins (approximately 10 μg in each lane) from different preparations were blotted with specific antibodies. Immunoreactive bands were visualized with peroxidase-conjugated anti-mouse or rabbit IgG antibody and an Enhanced Chemiluminescence kit (Amersham). Relative expression levels of the proteins were quantified by densitometric scanning analysis.

**RESULTS**

**Region-specific expression of Mash-1 and Prox-1 in the developing CNS**

Previous studies reported expression patterns of Mash-1 and Prox-1 during development in mouse (Lo et al., 1991; Guillmot and Joyner, 1993; Oliver et al., 1993). We extended these studies and examined their spatio-temporal regulation in more detail. Whole-mount in situ hybridization studies revealed that in the developing rat forebrain at E13.5, the Mash-1+ domain covered the ventral thalamus (VT), hypothalamus (HT) and ganglionic eminence (GE; Fig. 1A). Strong expression was also detected in the dorsal midbrain, where neurogenesis proceeds earlier than in the forebrain. In contrast, the dorsal thalamus (DT) and the primordia of the cerebral cortex (CC) were devoid of expression of Mash-1 at this stage. Thus, Mash-1-positive and -negative domains were found adjacent with sharp boundaries at the position between the DT and VT and between the CC and GE (arrowheads in Fig. 1A; also see Fig. 21). Immunocytochemical studies showed that the expression domains of Prox-1 followed the discrete patterns of Mash-1, which also demarcated sharp boundaries (Fig. 1D,E). We noticed that these characteristic expression patterns were reminiscent of the two brain-specific homeobox genes, Dlx-1 and Pax-6 (Bulfone et al., 1993; Stoykova and Gruss, 1994). Thus, the expression domains of Mash-1 and Prox-1 closely overlapped with that of Dlx-1 in the ventral forebrain (Fig. 1D-F,G-I), and were rather reciprocal to that of Pax-6 in the dorsal region, except that all of them overlapped each other in the VT (Fig. 1B,C). It is notable that these domains are known to correspond to important neuromeric compartments of the embryonic forebrain (Puelles and Rubenstein, 1993). Thus, the above results suggest that the region-specific expression patterns of Mash-1 and Prox-1 are closely correlated with the establishment of regional specificity of the developing brain. Furthermore, differential onsets of their expressions appeared to reflect distinct kinetics of neurogenesis among different domains of the CNS. The above results showing that the expression of Mash-1 and Prox-1 occurs earlier in the VT than in the DT are consistent with the fact that generation of the major neuronal population in the former generally precedes that in the latter (Angevine, 1970). Postmitotic neurons in the GE and HT are also known to be produced earlier than those in the cortical plate, except for the early-born Cajal-Retzius cells located in the pial surface of the CC (Altman and Bayer,
Consistently, strong expression of Prox-1 was seen to be coincident with the later onsets of neuronal differentiation in the DT and CC at E17.5 and E20.5 (Fig. 1J-L). These observations suggest that the spatio-temporal regulation of Mash-1 and Prox-1 is tightly associated with the mechanisms controlling the onset of neuronal differentiation.

Properties of Mash-1- and Prox-1-expressing cells in the developing neuroepithelium

Next we compared the expression of Mash-1 and Prox-1 with those of two other markers for specific neural cell lineages to characterize the properties of the expressing cells in the developing forebrain. Nestin is a class of intermediate filament proteins expressed in undifferentiated neuroepithelial cells in the developing CNS (Lendahl et al., 1990). We and others have shown that CNS stem cells abundantly express nestin (Redies et al., 1991; Nakafuku and Nakamura, 1995; Reynolds and Weiss, 1996). MAP2 is one of the earliest markers specific for postmitotic neurons. In rat embryos, most of the cells in the forebrain remain mitotically active until E11.5 (Frederiksen and McKay, 1988). At this stage, nestin was strongly expressed throughout the neuroepithelium (Fig. 2D), whereas only a few MAP2-positive (MAP2+) cells were detectable (Fig. 2C, arrows). In the same region, Mash-1 and Prox-1 were localized in nuclei of some clusters of cells (Fig. 2A,B, arrows). A higher magnification clearly showed that these cells emerged earlier than MAP2+ neurons and constituted a minor population among nestin+ neuroepithelial cells (Fig. 2E-H). As development proceeded to E14.5, a large number of neurons differentiated in the mantle zone (MZ) in the basal forebrain (Fig. 2K,O). Nestin+ cells were found mainly in the ventricular zone (VZ) and also some in the subventricular zone (SVZ), where proliferating precursors resided (Fig. 2L,P). At this stage, many Mash-1+ cells emerged in the VZ and SVZ, which appeared to overlap with the nestin+ area (Fig. 2M,P). At higher magnification, a small scattered population was also detected in the inner region of the MZ in the GE (Fig. 2M, arrow). On the other hand, Prox-1+ cells were mainly detected in the SVZ, where they appeared to overlap with Mash-1+ cells (Fig. 2N). Furthermore, some Prox-1+ cells spread further into the MZ, where its expression partly overlapped with MAP2.

Next we examined whether Mash-1+ cells are mitotically active. Proliferating cells were labeled with BrdU and detected by double-staining (Fig. 2Q,R). When focused on the GE, 47±2% \( (n=4) \) of the cells located in the VZ and SVZ incorporated BrdU after a 3-hour incorporation period. Under these conditions, 36±3% \( (n=4) \) of Mash-1+ cells were labeled with BrdU, whereas 56±5% \( (n=4) \) of BrdU+ cells expressed

Fig. 1. (A-C) Expression domains of Mash-1 in the developing CNS (A) were compared with those of Dlx-1 (B) and Pax-6 (C) by whole-mount in situ hybridization. Side views of the dissected E13.5 embryonic brain are shown. The arrowheads indicate the positions of sharp expression boundaries shared by these genes. (D-L) Expression patterns of Mash-1 (D,G), Prox-1 (E,H,I-L) and Dxl-1 (F,J) in the developing rat brain were examined by immunohistochemistry on parasagittal (D-I) and coronal (J-L) sections of the forebrain at E14.5 (D,I), E17.5 (J) and E20.5 (K,L). G-I are higher magnification views of the areas boxed in D-F, respectively, in which the distribution of Mash-1+ and Prox-1+ cells was compared with that of Dlx-1+ cells. The arrowheads indicate the positions of sharp expression boundaries. In L, late-generated Prox-1+ cells in the ventricular zone of the cerebral cortex boxed in K are shown at higher magnification (see text for details). CC, cerebral cortex; CP, cortical plate; DT, dorsal thalamus; GE, ganglionic eminence; HT, hypothalamus; MB, midbrain; PT, pretectum; VT, ventral thalamus; VZ, ventricular zone. Bars, 1 mm (A-F); 200 \( \mu \)m (G-L); 5 mm (J,K).
Mash-1. Considering the 3-hour labeling period and cell cycle kinetics of proliferating cell populations in the GE (Bhide, 1996), the majority of the Mash-1+ cells in the VZ and SVZ were mitotically active at this particular stage.

Similar expression profiles of Mash-1 and Prox-1 were also observed in the developing spinal cord. The domain of Mash-1+ cells was located at the ventricular side and reciprocal to that of MAP2+ cells (Fig. 3A,C). The expression of Prox-1 overlapped with both of these domains, constituting a narrow band between the VZ and MZ. The sequential distributions of the above three markers were more clearly seen at higher magnification (Fig. 3E-H). Consistent with a previous report (Saito et al., 1996), we detected cells labeled by both anti-Mash-1 and anti-BrdU (Fig. 3H,I) in the VZ, where double-labeled cells corresponded to 24±2% (n=4) of the total Mash-1+ cells. Moreover, Prox-1+/BrdU+ cells were also detectable...
Table 1. Properties of Mash-1- and Prox-1-expressing cells

<table>
<thead>
<tr>
<th>Marker</th>
<th>% of positive cells in total cell number</th>
<th>Co-expression of other markers (% fraction in total positive cells)</th>
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<tr>
<td></td>
<td>Mash-1</td>
<td>Prox-1</td>
</tr>
<tr>
<td>Mash-1</td>
<td>17.8±3.5</td>
<td>NA</td>
</tr>
<tr>
<td>Prox-1</td>
<td>9.8±1.2</td>
<td>87.7±3.4</td>
</tr>
<tr>
<td>Nestin</td>
<td>38.5±5.4</td>
<td>31.2±4.5</td>
</tr>
<tr>
<td>MAP2</td>
<td>48.7±4.9</td>
<td>0±0.3</td>
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Primary culture was established from the neuroepithelium of the ganglionic eminence of E14.5 rat embryos. After seeding dissociated single cells onto glass chambers coated with poly-D-lysine (100 μg/ml), double immunostaining was performed with various combinations of specific antibodies for Mash-1, Prox-1, nestin and MAP2. The percentages of positive cells for each marker in the total cell number are shown, together with the fractions of the cells that co-expressed other markers, as percentages in the total number of positive cells.

Values are the means ± s.d. (n=3-5), in which at least 20 non-overlapping visual fields and 1000 independent cells were scored.

NA, not applicable; <0.2, less than 0.2%.

Mash-1 and Prox-1 during development of the CNS. As shown in Fig. 4A, we speculate that at early stages of development, neither Mash-1 nor Prox-1 was expressed in nestin+ undifferentiated neuroepithelial cells. At the onset of their differentiation, Mash-1 expression is induced first, and is followed by the expression of Prox-1 and down-regulation of nestin. Nestin and Prox-1 appear to be mutually exclusive at the single cell level. Importantly, Mash-1+/Prox-1+ cells are mitotically active, although to what extent they can divide is unknown. With further progression of differentiation, Mash-1 expression is extinguished, but expression of Prox-1 remains to some extent in newly forming neurons. According to this model, Mash-1+/Prox-1+ cells are defined as transient proliferative precursors that are molecularly distinct from stem cells and subsequently differentiate into neurons, which we call secondary precursors. Since gliogenesis proceeds only at later stages of neural development, it remains undetermined at present whether this particular cell population gives rise to glia as well as to neurons. It should be also noted that the above results do not provide direct evidence that the same sequence indeed occurs within a single cell lineage in vivo.

Correlation of Mash-1 and Prox-1 with initial differentiation of neural stem cells

Although the above studies revealed the unique nature of Mash-1- and Prox-1- expressing cells, their precise lineage relationship to stem cells was still unknown. To address this issue, we took advantage of an in vitro culture system, in which self-renewal and differentiation of stem cells can be manipulated. Fig. 5A illustrates a detail of the culture protocol used in this study. The neuroepithelium of the E11.5 forebrain was dissected out, and primary cultures were established. When the cells were maintained under monolayer conditions in a serum-free defined medium (M1), total numbers of viable

Fig. 3. Expression of Mash-1 and Prox-1 in the developing spinal cord. Localizations of cells that expressed Mash-1 (A,E,I) and Prox-1 (B,F,J), MAP2 (C,G) were compared on adjacent transverse sections of the spinal cord of E14.5 rat embryos. E-H are higher magnification views of the areas boxed in A-D. In D, H and I, co-localization of Mash-1 (blue) and BrdU antigen (brown) was visualized (arrows in I). Expression of Prox-1 (brown) in nuclei of BrdU-labeled cells (blue) was shown in J (arrows). In E-H, relative distributions of stained cells are indicated by the brackets. MN, motor neurons; MZ, mantle zone; P, pial surface; RP, roof plate; V, ventricle; VZ, ventricular zone. Bars, 200 μm (A-D); 50 μm (E-H); 20 μm (IJ).
cells remained relatively constant (Fig. 5B, filled squares), although BrdU-labeling studies revealed the presence of a minor population of dividing cells for the initial 2-3 days (not shown). Under these conditions, MAP2+ neurons became detectable at day 3, and thereafter their numbers gradually increased in the following 3 days (Fig. 6B). Glial differentiation required an additional 5-10 days (not shown). In contrast, when the same population was cultured in suspension in the presence of EGF and bFGF (A1 in Fig. 5A), a small fraction of proliferating cells (1 cell out of 430±45, n=5) could be recovered. These cells, even when cultured as individual single cells, continued cell division (calculated doubling time was 16.2±0.7 hours, n=5; Fig. 5B, filled circles), and formed large cell aggregates termed neurospheres, as first described by Reynolds et al. (1992) and subsequently characterized in detail (Vescovi et al., 1993; Reynolds and Weiss, 1996; Gross et al., 1996). The majority of cells in these aggregates were nestin+, indicating that they retained properties of undifferentiated cells (Fig. 5C,D). When a single neurosphere was transferred to monolayer culture (M2), the cells stopped growing (Fig. 5B, open squares), and spread out on the culture surface (Fig. 5E). Importantly, under appropriate conditions we could detect generation of multiple cell types from a single neurosphere, including neurons, astrocytes and oligodendrocytes (Fig. 5F-H). On the other hand, when single-cell suspensions were made from these spheres and re-plated under aggregation conditions (A2), the cells continued proliferation, again forming neurospheres (Fig. 5B, open circles). In this sense, the cells first recovered as neurospheres fulfilled the criteria of multipotential CNS stem cells in that they remained undifferentiated while undergoing multiple rounds of self-renewal, whereas they could give rise to neurons and glia under particular conditions. Thus, this defined culture system allowed us to manipulate two distinct cell fates of stem cells. One is the fate for continuing self-renewal, and the other is the fate for undergoing differentiation into neurons and glia.

Using these cultures, the expression patterns of Mash-1 and Prox-1 were examined by western blotting. When primary cells were directly placed in monolayer culture (M1), they did not express either of these proteins at day 1. However, transient induction of Mash-1 and Prox-1 was detected at day 3 (Fig. 6A). Immunostaining of single cells detected the induction of Mash-1+ cells at days 1 and 2 more sensitively, and clearly demonstrated that it preceded the emergence of MAP2+ neurons (Fig. 6B). On the other hand, expression of neither Mash-1 nor Prox-1 was detected when stem cells were maintained in aggregation culture (Fig. 6A,A1). Both markers remained negative even when the cells were subjected to the second cycle of aggregation culture (Fig. 6A,A2). These results indicated that self-renewing stem cells do not express either Mash-1 or Prox-1. However, when self-renewing cells were transferred to monolayer culture conditions, Mash-1 and Prox-1 were again induced at day 1 through day 3 (Fig. 6A,M2). These results strongly suggest that Mash-1+ and Prox-1+ cells show the direct precursor-product relationship with self-renewing stem cells. Furthermore, the emergence of these cells closely coincided with an initial step of differentiation of neural stem cells.

**Functional roles of Mash-1 and Prox-1 in neural stem cells**

A further important question is whether the sequential differentiation process proposed in Fig. 4B indeed occurs in a single cell lineage. To address this issue, we used the immortalized clonal cell line MNS-70. As described previously (Nakagawa et al., 1996), this cell line exhibits many of the properties of CNS stem cells, and expressed nestin while they were maintained in monolayer (Fig. 7A). To induce differentiation, the cells were allowed to form cell
aggregates reminiscent of the above mentioned neurospheres (for details, see Nakafuku and Nakamura, 1995). Under these conditions, the majority of the cells still remained undifferentiated, although the fraction of nestin+ cells decreased to about 34%, and the intensity of nestin staining became much weaker in individual cells. Subsequently, the cells were subjected to differentiation culture, in which a marked induction of neurons and glia was observed (Fig. 7A). Western blot analyses using the above cultures detected no significant expression of Mash-1 or Prox-1 in monolayer cells (Fig. 7C). In aggregation culture, however, their levels were markedly elevated. On proceeding to differentiation culture, the levels of Mash-1 and Prox-1 became about 20-fold less than those in aggregation culture. Immunocytochemical studies also revealed the transient accumulation of Mash-1+ and Prox-1+ cells (Fig. 7B). In aggregation culture, most of the cells became positive for Mash-1 (85.6±6.9%, n=5) and Prox-1 (78.5±4.1%, n=5). On the other hand, the percentages of labeled cells rather decreased in differentiation culture. This transient pattern was in clear contrast to the induction of neuronal and glial cell markers, the levels of which were highest in differentiation culture (Fig. 7A). It is important to note here that the majority of the cells in aggregation culture expressed Mash-1 and Prox-1, whereas the most of the cells in differentiation culture expressed either neuronal or glial cell makers. These properties of the clonal cell line, which was supposed to be derived from a single stem cell, strongly suggested that the sequential induction events, i.e. early generation of Mash-1+/Prox-1+ cells from nestin+ stem cells and subsequent differentiation into neurons and glia, indeed occur within a single cell lineage.

By taking advantage of the above characteristics, we next established sublines in which Mash-1 or Prox-1 was constitutively expressed. Fig. 8A shows the sublines which expressed high levels of Mash-1 proteins in monolayer culture. Under these conditions, expression of Mash-1 was below detectable levels in the parental cells and control sublines. In the representative M1-12 and M1-13 cells, high levels of Mash-1 proteins were maintained in aggregation culture, which were subsequently extinguished in the cells in differentiation culture (Fig. 8B), despite the fact that constitutive high levels of Mash-1 mRNA from the transgene were detected in those cells (data not shown). The reasons for these phenomena are not clear at present, but expression of Mash-1 proteins may be regulated at the post-transcriptional level. On examining these sublines, we found that constitutive expression of Mash-1 caused characteristic phenotypic changes of MNS-70 cells. As described above, down-regulation of nestin and induction of Prox-1 were closely associated with Mash-1, both in vivo and in vitro. Consistently, it was found that the expression of nestin was extinguished in the Mash-1-expressing sublines. In the representative subline M1-12, the expression of nestin was detectable under none of the culture conditions, whereas it was...
successively down-regulated in the control subline H1-1 (Fig. 8C). Furthermore, the expression of Prox-1 became constitutive in the sublines. In M1-12 and M1-13 cells, the levels of Prox-1 proteins remained high in aggregation culture, and were subsequently decreased in differentiation culture, the pattern of which paralleled that of Mash-1 (Fig. 8D). Immunostaining of single cells demonstrated that the majority of the cells in monolayer cultures of M1-12 and M1-13 expressed Prox-1 but not nestin, demonstrating that the reciprocal regulation of these proteins by Mash-1 simultaneously occurred in the same cells (data not shown). In separate experiments, we also established sublines in which Prox-1 was constitutively expressed. None of these sublines showed altered expression of nestin or Mash-1 (T. M. and M. N., unpublished). Thus, we conclude that Mash-1 functions, either directly or indirectly, as an upstream regulator for the expression of nestin and Prox-1, thereby contributing to the early steps of differentiation in neural stem cells.

We next asked whether the Mash-1-expressing cells maintain multipotentiality or are already committed to particular cell lineages (Fig. 8E). In monolayer, M1-12 cells did not express either MAP2, GFAP or GD3-ganglioside at detectable levels, indicating that forced expression of Mash-1 in MNS-70 did not directly induce terminal differentiation of neuronal or glial cell lineage. We found, however, M1-12 cells could give rise to both neurons and glia when they were conditionally induced to differentiate like their parental cells. The five other Mash-1-expressing sublines shown in Fig. 8A also exhibited similar multipotential properties. These results suggested that terminal differentiation of neurons and glia involves additional steps other than the induction of Mash-1 and Prox-1 in MNS-70 cells.

**Defects in neurogenesis in Mash-1 knock-out mice**

To further explore functional roles of Mash-1 in vivo, we next examined the mutant mouse strain in which the Mash-1 locus was genetically disrupted (Guillemot et al., 1993). Although developmental defects in the PNS of the mutant have been characterized (Guillemot et al., 1993; Sommer et al., 1995; Cau et al., 1997), phenotypes in the developing CNS has not yet been examined in detail. As in the case of rat embryos, Mash-1 was strongly expressed in specific subdomains in the mouse forebrain at E12.5 (roughly
corresponds to E14.5 in rats; see Figs 1, 2) including the dorsal part of the epithalamus (ET), VT and the ventral floor region of the HT (Fig. 9C). We found that the shape of the neural tube was severely deformed in these three regions of the mutant embryos (Fig. 9A,B). Higher magnification views revealed that the neuroepithelium was expanded in the ET and VT (about 1.5- to 2-fold thicker than those in the wild-type embryos; Fig. 9D,E), and in some cases a protrusion toward the ventricle was evident (arrow in Fig. 9B,E). A similar but less prominent expansion was also evident in the ventral aspect of the HT (Fig. 9F). On the other hand, MAP2 staining, which identified postmitotic neurons in the MZ of the neuroepithelium, revealed that the MZ was largely reduced in size or almost missing in the above three regions (Fig. 9D-F). We also noticed a similar hypomorphic phenotype of the MZ in the median part of the GE and the dorsal aspect of the neural tube throughout the midbrain to the spinal cord (S. Casarosa et al., 1999). Thus, the fact that the neuroepithelium itself was enlarged but the MZ was shrunken indicated that proliferative cell populations in the VZ were expanded in the mutant brain. It was also noted that Prox-1+ cells were also reduced in number or almost completely lost in those affected domains (Fig. 9B,G), which supported the idea that Mash-1 is involved in regulating Prox-1 in vivo. The lack of Prox-1 expression in the cells that accumulated in the mutant neuroepithelium further suggested that they maintained properties of self-renewing stem cells. Consistently, the majority of them were stained by anti-nestin antibody (data not shown). These phenotypes were in clear contrast to the apparent normal histogenesis in the adjacent domains, i.e. the DT and the dorsal portion of the HT, where the formation of the MZ remained intact. Expression of Prox-1 was also detectable in those areas. Thus, defects in the mutant forebrain were highly specific to the domains where Mash-1 was strongly expressed in the wild-type embryos at this stage. The evidence that the VZ was expanded at the expense of the MZ in the mutant brain supported the idea that stem cells cannot appropriately differentiate into neurons or express Prox-1 in the absence of normal functions of Mash-1. Thus, the abnormalities observed here provided
strong evidence that Mash-1 plays an essential role for differentiation of stem cells in vivo.

**DISCUSSION**

**Mash-1 and Prox-1 as molecular markers for transient precursors in the CNS**

During development in vertebrates, multipotent stem cells give rise to diverse cell types present in the adult CNS (Temple and Qian, 1996; McKay, 1997). Recent studies have demonstrated that stem cells generate neurons and glia through a multi-step process involving many types of intermediate precursors. These precursors are defined as cells that have more restricted self-renewing capacities and/or differentiation potentials than stem cells (Kilpatrick, et al., 1995; Temple and Qian, 1996; Lillien, 1998). However, many of them are transient cell types, and therefore their lineage relationships still remain poorly understood. Such issues of identities and lineage relationships among diverse precursors is one of the fundamental questions in developmental biology (Hall and Watt, 1989).

The aim of this study was to identify transient precursors present in the developing CNS by using defined molecular markers. Here we present evidence that the bHLH-type transcription factor Mash-1 and the homeodomain-containing protein Prox-1 define a transient precursor that is molecularly distinct from the CNS stem cells. The expression patterns of those markers both in vivo (Figs 1-3) and in primary culture of neuroepithelial cells (Figs 5, 6) suggested the model that Mash-1+ and Prox-1+ cells originate from nestin+ stem cells, and subsequently give rise to terminally differentiated neurons and glia (see Fig. 4B). Recent studies have shown, however, that nestin is expressed in different types of cells in immature stages, and hence is not a definitive marker for stem cells (Zimmerman et al., 1994). In this study, therefore, we defined the properties of neural stem cells by their ability of self-renewal and their differentiation potential (Figs 5, 6). Under our culture conditions, self-renewing stem cells did indeed express nestin, and down-regulation of nestin was closely coupled with the emergence of Mash-1 and Prox-1, which further led to the generation of neurons and glia. At present, however, the transient nature of the expression of Mash-1 and Prox-1 precludes direct proof that the apparently sequential events of cellular differentiation do indeed occur within a single cell lineage. Nevertheless, analysis of the stem cell-derived clonal cell line MNS-70 strongly supported our model (Figs 7, 8). Thus, undifferentiated MNS-70 cells expressed nestin but not Mash-1 or Prox-1. Upon induction of differentiation, however, the majority of them transiently expressed Mash-1 and Prox-1, and subsequently generated neurons and glia. Furthermore, forced expression of Mash-1 in nestin+ MNS-70 cells caused the induction of Prox-1 and down-regulation of nestin in each single cell. In addition, we

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Fig. 9. Defective neurogenesis in the forebrain of the Mash-1 mutant mice. The expression domains of MAP2 (A,A’,D-F,D’-F’) and Prox-1 (B,B’,G,G’) in the developing forebrain at E12.5 were compared between the Mash-1 mutant (A-G) and wild-type (A’-G’) mice. The expression of Mash-1 in the wild-type embryos at the same stage was shown in C. The arrowheads in each panel indicate the boundaries of discrete neuromeric compartments of the developing forebrain. The arrows in B and E indicate a protrusion of the neuroepithelium at the dorsal part of the VT. The arrowheads in F,F’G,G’ mark the junction between the ventral and dorsal parts of the HT, where the formation of the MZ and expression of Prox-1 were missing in the former, whereas those remained intact in the latter. Abbreviations are the same as in Figs 1 and 2. Bars, 400 μm (A-C,A’,B’); 200 μm (D-G,D’-G’).
showed that differentiation of neurons and expression of Prox-1 were prevented by the loss-of-function mutation of Mash-1. This in vivo evidence strongly supported the idea that Mash-1 has a causal relationship with both the induction of Prox-1 and neuronal differentiation in the same cell lineage in the developing neuroepithelium. Importantly, Mash-1+/Prox-1+ precursors were found throughout the developing CNS, and therefore, successive steps of cellular differentiation proposed in this study are likely to be a general phenomenon during neural development.

We showed that, like self-renewing stem cells, Mash-1+/Prox-1+ precursor cells were mitotically active in vivo. However, both in the forebrain and spinal cord at E14.5, less than 10% of the total cells were Mash-1/Prox-1 double-positive, and the fraction did not significantly change until E16.5 and decreased thereafter (T. M. and M. N., unpublished). Furthermore, when self-renewing stem cells were allowed to differentiate in vitro, Mash-1 and Prox-1 were induced only in a transient manner and extinguished in terminally differentiated neurons and glia. Trials for in vitro expansion of self-renewing Mash-1+/Prox-1+ cells by using various growth factors were not successful in those cultures. However, the fact that Mash-1 and Prox-1 are nuclear proteins precluded the isolation of a pure population of living Mash-1+/Prox-1+ cells, and hence we could not directly examine their proliferative properties. Thus, it is still possible that these cells are able to continue multiple rounds of self-renewal under particular conditions. Nevertheless, the above observations strongly suggested that Mash-1+/Prox-1+ cells are transient precursors, and undergo only limited cycles of cell division both in vivo and in vitro.

Roles of Mash-1 and Prox-1 in early steps of differentiation in neural stem cells

During the early stage of development, neural stem cells have to choose two alternative cell fates; one is the fate for continuous self-renewal in keeping their multipotentiality, and the other is the fate committed to differentiation pathways towards the generation of neuronal and glial progeny. Control of this step is a critical part of the highly organized morphogenesis of the CNS. In this study we show that the induction of Mash-1 and Prox-1 in stem cells appears to coincide with the timing of this control event. Furthermore, we provide evidence that Mash-1 in fact participates in promoting an early step of differentiation of neural stem cells. When Mash-1 was constitutively expressed in the stem-cell derived cell line MNS-70, the expression of Prox-1 was induced, and conversely the expression of the stem cell marker nestin was extinguished. On the other hand, forced expression of Prox-1 did not alter the expression of either Mash-1 or nestin. Thus, Mash-1 appears to function upstream of Prox-1 and nestin, which is consistent with their spatial and temporal expression patterns in vivo. Furthermore, we also present evidence that Mash-1 plays vital roles in both the differentiation of neurons and the proper expression of Prox-1 in vivo. We found that the MZ containing MAP2+ neurons was severely reduced in size, and instead the VZ, where stem cells are supposed to reside, was expanded in specific regions of the developing brain of Mash-1 knock-out mice (Fig. 9). Consistently, the expression of Prox-1 was decreased or lost in those affected areas in mutant mice. It is interesting to note that in Drosophila, expression of prospero depends upon the AS-C genes in neuroblasts and GMCs (Doe and Technau, 1993; Hirata et al., 1995). Thus, our results showing Mash-1-dependent induction of Prox-1 imply an analogous functional relationship of these genes with their fly counterparts. Taken together, these phenotypic defects of Mash-1 mutants in vivo strongly supported our model obtained from in vitro cell culture studies. It remains to be determined, however, whether or not the transition from stem cells to Mash-1+/Prox-1+ precursors is irreversible. In addition, further studies are needed to clarify the functions of Prox-1 in neural development.

Analogous and distinct roles of Mash-1 in the CNS and PNS

As discussed above, we demonstrate that Mash-1 plays a role in a transient precursor cell in the CNS. It is notable that in the PNS, Mash-1 is also expressed in precursor cells committed into the lineage of autonomic neurons (Lo et al., 1991). Consistently, the analysis of the Mash-1 mutant mice revealed that Mash-1 plays a critical role for appropriate differentiation of autonomic neurons in vivo (Guillemot et al., 1993; Sommer et al., 1995). Furthermore, a recent study has demonstrated that constitutive expression of Mash-1 in neural crest stem cells can directly lead to neuronal differentiation in vitro (Lo et al., 1998). Thus, Mash-1 in the PNS appears to promote both commitment and differentiation of a particular (in this case autonomic) neuronal cell type. On the other hand, our results indicate that in the CNS stem cells, Mash-1 expression did not directly result in neuronal lineage-specific differentiation. Although transient induction of Mash-1 occurred in most of the cells in MNS-70, they subsequently generated both neurons and glia (Fig. 7A,B). Furthermore, sublines of MNS-70 that constitutively expressed Mash-1 did not express neuronal or glial cell markers in monolayer. On the other hand, they could still give rise to neurons, astrocytes and oligodendrocytes upon conditional induction like their parental cells (Fig. 8E). Thus, the Mash-1+ precursors identified in this study are phenotypically distinct from those found in the PNS in that they still retain multipotentiality. In the lineage of olfactory neurons, Mash-1 is also proposed to be expressed in transiting-amplifying precursors (Gordon et al., 1995; Cau et al., 1997). In this case, however, their lineage-relationships with self-renewing stem cells have not yet been clarified, and it is still an open question whether Mash-1 functions to restrict the lineage and differentiation of olfactory neurons.

Specific and redundant functions of bHLH genes in brain development

In this study, we describe the phenotypes caused by a loss-of-function mutation of Mash-1 in the developing mouse brain. In the mutant forebrain at E12.5, we found severe hypomorph or loss of the MZ in specific regions including the ET, VT, and HT, where Mash-1 was strongly expressed in normal embryos at that stage (Fig. 9). However, it was also noted that defects of the MZ was much less prominent in some Mash-1+ regions such as the lateral GE and septum, although a loss of specific neuronal subpopulations was still detectable in those regions (S. Casarosa et al., 1999). Prox-1 expression was also detected in those regions at E12.5 (T. M. and M. N., unpublished). This may suggest the possibility that other related bHLH genes complement the loss of function of Mash-1 in some specific
subdomains of the forebrain. In fact, an increasing number of bHLH-type transcription factors has been identified, and some of them show overlapping expression patterns with Mash-1 in the developing CNS (Gradwohl et al., 1996; Lee, 1997). In MNS-70 cells, multiple bHLH factors other than Mash-1, including Math-1 and NeuroD, were expressed at detectable levels. Unlike Mash-1, however, overexpression of NeuroD did not result in down-regulation of nestin or induction of Prox-1 (M. T. and M. N., unpublished). Thus, at least in this particular cell line, Mash-1 exhibited specific functions.

Significance of multiple precursors and multiple molecular regulators

The present study has left open the issue of the biological significance of multiple precursor cell types during neurogenesis of the CNS. One tenable idea is that distinct precursors expressing different sets of regulatory genes are targets for distinct environmental signals. In fact, recent studies have demonstrated that commitment and differentiation of neurons and glia are controlled by multiple signaling factors (see McKay, 1997, for a review). In particular, platelet-derived growth factor has been proposed to promote neuronal differentiation, whereas bone morphogenetic protein 2 (BMP2) and ciliary neurotrophic factor specifically stimulate astroglial differentiation (John et al., 1996; Gross et al., 1996; Williams et al., 1997). In the PNS, BMP2 was shown to induce the expression of Mash-1 in neural crest stem cells, and promoted differentiation of autonomic neurons (Shah et al., 1996). However, in our culture system of CNS stem cells, none of the above factors could directly induce the expression of Mash-1 or Prox-1 (T. M. and M. N., unpublished). On the other hand, bFGF and EGF, which acted as potent mitogens for self-renewing stem cells, appeared to inhibit the induction of Mash-1 and Prox-1 (see Fig. 5). Thus, it still remains unknown what environmental factor(s) is responsible for determining the onset of the expression of Mash-1 in self-renewing stem cells. Alternatively, induction of Mash-1 may be under regulation by cell-cell interactions through the Notch-signaling pathway as demonstrated in other bHLH genes in Xenopus and Drosophila (reviewed in Kageyama and Nakanishi, 1997). Consistent with this idea, a recent report has described that targeted disruption of Notch1 and its downstream signaling component RBP-Jk in mice resulted in aberrant expression of Mash-1 in the CNS (de la Pompa et al., 1997).

Spatio-temporal regulation of differentiation of neural stem cells

Based on the above evidence, the present study explains why the time at which the expression of Mash-1 and Prox-1 is first turned on is highly variable in distinct regions of the developing CNS (Lo et al., 1991; Guillemot and Joyner, 1993; Oliver et al., 1993). These distinct times of onset are likely to reflect distinct onsets of differentiation of stem cells. This idea is supported by our observations that their spatio-temporal expression patterns are correlated with the onset and kinetics of neuronal differentiation in various regions (Figs 1, 2). Furthermore, we showed that the expression domains of Mash-1 and Prox-1 closely overlapped that of Dlx-1 and were reciprocal to that of Pax-6 in the rat forebrain at E13.5 (Fig. 1). It is striking that these genes shared important neuromeric boundaries in the developing forebrain (Puelles and Rubenstein, 1993; Bulfone et al., 1993; Stoykova and Gruss, 1994). These observations raise the intriguing possibility that the mechanisms controlling differentiation of neural stem cells are closely coupled with those underlying the preceding regional specification of the developing neuroepithelium. In fact, a recent study has reported that several distinct enhancer elements are responsible for regulating the expression of Mash-1 in different regions of the developing CNS (Verma-Kurvari et al., 1998). Further studies to elucidate the molecular mechanisms that coordinate the regional specification and differentiation of neural stem cells will facilitate better understanding of early development of the vertebrate CNS.

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